Microbiota and volatilome profile of fresh and chill-stored deepwater rose shrimp (Parapenaeus longirostris)

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ABSTRACT

Bacterial communities and Volatile Organic Compounds (VOCs) profile of deepwater rose shrimp (*Parapenaeus longirostris*) stored at 0°C (ice) and 4°C were investigated using 16S amplicon based sequencing and Solid phase micro-extraction (SPME) - Gas chromatography/mass spectrometry (GC/MS), respectively. The shelf-life of shrimps determined by sensory assessment was 5 and 2 days at 0°C and 4°C, respectively. Based on 16S analysis (culture-independed), the initial microbiota of shrimps mainly consists of *Photobacterium*, *Candidatus Hepatoplasma*, *Psychrobacter*, *Acinetobacter* and *Delftia*. *Psychrobacter* and *Carnobacterium* dominated during storage at both temperatures. *Psychrobacter* was the most dominant taxon at the end of shelf-life of chill-stored shrimps. A minor microbial population composed by *Brevundimonas*, *Stenotrophomonas*, *Staphylococcus*, *Legionella*, *Acinetobacter*, *Bacillus*, *Escherichia-Shigella*, *Enterococcus*, *Enterobacter*, *Klebsiella* was also detected. Those taxa may be originated from the environment due to an inadequate hygienic practice during fishing, handling and icing. VOCs such as ethanol, 3-methyl-1-butanol, 2-ethyl-1-hexanol, 3-hydroxy-2-butanone, indole etc., were found to be associated with shrimps at 4°C, while acetone and dimethyl sulfide with shrimps in ice. Some VOCs, from microbial or chemical origin, increased in shrimps either at 0°C (i.e. 1-octen-3-ol, *trans*-2-octenal) or at 4°C (i.e. 3-methyl-1-butanol, indole), while 2-methylbutanal and 3-methylbutanal increased in both temperatures. A positive correlation between *Psychrobacter* with 2-ethyl-1-hexanol and *Carnobacterium* with 3-methyl-1-butanol was also observed. Concluding, we suggest the reinforcement of Good Hygiene Practices on fishing boats during fishing/handling, the rapid onboard icing and keeping shrimps iced avoiding even small increase of storage temperature that affects quality parameters (e.g. microbial

population level, synthesis of microbiota, VOCs profile) in order to provide a product of the highest quality and safety in the market.

Keywords: crustaceans, shrimps, spoilage, microbiota, 16S Next Generation Sequencing, Volatile Organic Compounds

1. Introduction

Crustaceans are traded and consumed in million tons around the world. The deepwater rose shrimp (*Parapenaeus longirostris*, Lucas 1846) is an important commercial species of the coasts of Italy, Spain, France, Algeria, Tunisia, Greece and Turkey, with Italy (4631 tons) and Spain (4237 tons) to be the countries with the largest catches (FAO, 2019). *P. longirostris* is one of the most landed and highly consumed shrimp species in Greece, where it is consumed as unprocessed. However, rose shrimp is extremely sensitive to deterioration even when it is stored in ice (shelf-life 6 days, Mendes, Gonçalves, Pestana & Pestana, 2005).

Despite the spoilage of shrimp begins with the appearance of melanosis (Ashie, Smith & Simpson, 1996; Lopez-Caballero, Martinez-Alvarez, Gomez-Guillen & Montero, 2006), microorganisms grow quickly and deteriorate its quality. Fresh shrimp spoils due to the rich content of protein, non-protein nitrogen compounds and other nutrients that can allow microbial growth (Don, Xavier, Devi, Nayak & Kannuchamy, 2018; Licciardello, Kharchoufi, Muratore & Restuccia, 2018). Microbial spoilage of shrimp begins immediately after catch and the microbial growth can be controlled by rapid icing of shrimps (Heinsz, Harrison & Leiting, 1988). A consortium of bacteria, the so-called Specific Spoilage Organisms (SSOs), is a small part of the initial total microbiota which grows faster over the other members of the

total microbiota and produces metabolites responsible for the development of off-odors and off-flavors during storage resulting the organoleptic rejection of the product (Gram & Huss, 1996). The domination of particular microorganisms or SSOs depends on various factors such as fish species, geographical origin, synthesis of the initial fish microbiota, microbial interactions, contamination during fishing, handling, storage and distribution in combination with the applied storage conditions (Boziaris & Parlapani, 2016; Parlapani et al., 2018b). The type of the microorganisms that prevail during storage determines the type of fish spoilage e.g. the production of Volatile Organic Compounds (VOCs) and the development of specific off-odors and off-flavors (Boziaris & Parlapani, 2016).

The use of 16S Next Generation Sequencing is able to reveal cultivable and non-cultivable bacterial genera directly from the sample which they may be involved in spoilage of shrimps (Yang, Xie & Qian, 2017) and other seafood (Chaillou et al. 2015; Kuuliala et al. 2018; Parlapani et al. 2018a; 2018b; 2019; Zotta, Parente, Ianniello, De Filippis & Ricciardi, 2019). In addition, microorganisms may be associated with hygiene conditions during fishing, icing, landing and distribution or potential pathogens coming from various sources of contamination can be also highlighted (Parlapani et al. 2018b). The derived information can be used to implement preventive measures for the improvement of microbial quality and safety of fish during handling and storage.

Despite the increasing consumption of deepwater rose shrimp, there is no study, to our knowledge, about bacterial communities and volatile compounds production during chill storage. The aim of this study was: (i) to determine microbiota through 16S amplicon sequencing, (ii) to investigate the VOCs profile using SPME-GC/MS and (iii) to correlate the volatilome with microbiome and sensory score for

chill-stored shrimps, in order to reveal the initial microbiota of shrimps, the microbiota changes during storage and the produced VOCs at the time point the particular microorganisms dominate the shrimp causing spoilage.

2. Materials and Methods

2.1 Shrimps provision, handling and storage

Two different batches of the deepwater rose shrimp (*P. longirostris*), that were caught by trawl fishery from Strymonikos Gulf (mean weight±sd: 12.3±3.0g, 72m depth, F/V Athanassios) in April (10-04-2017, 17-04-2017) were packaged in insulated boxes with melted ice (0°C) and transferred to the Laboratory of Marketing and Technology of Aquatic Products and Foods (Department of Ichthyology and Aquatic Environment, University of Thessaly, Volos, Greece) within four hours from capture. The insulated boxes containing 1kg of shrimps each, were stored in incubators operating at two different temperature conditions; 0°C (shrimps with melted ice replaced every day) and 4°C.

2.2 Evaluation of shrimp's shelf-life

Sensory evaluation was performed by 5 trained panelists, who evaluated the appearance of the shell (bright colours, slight blackening on the head, blackening on head and body), and flesh odor (fresh, marine, musty, ammoniacal, sour, putrid), according to FAO/WHO (1999). The sensory attributes rating were scored using a 5 to 1 scale with 5 (excellent), 4 (good), 3 (acceptable), 2 (unacceptable) and 1 (spoiled) scores. A score of 3 was considered as the score for minimum acceptability and the time point that average score was below 3 (which means that at least one out of the five panelists scored with 2) was considered as the rejection time point.

2.3 Microbiological analysis

Ten grams (10 g) flesh from 10 shrimp individuals were aseptically transferred into stomacher bags with 90 mL MRD (Maximum Recovery Diluent, 0.1% w/v peptone, 0.85% w/v NaCl) and homogenized for 2 min using a Stomacher (Bug Mixer, Interscience, London, UK). Using the spread plate technique, 0.1 mL from 10-fold serial dilutions were used in order to enumerate the total microbial population as aerobic plate counts (APC) on TSA (Tryptone Soy Agar) and *Pseudomonas* on cetrimide-fucidin-cephaloridine agar (CFC) after incubation at 25°C for 48 h. Using the pour plating method with overlay technique, 1 mL of serial dilutions in MRD was used for the enumeration of counts on Iron Agar (IA) by counting only the black colonies (H_2S producing bacteria) after incubation at 25°C for 72 h, Lactic Acid Bacteria (LAB) on De Man, Rogosa, Sharpe agar (MRS) after incubation at 25°C for 72 h and counts on Violet Red Bile Glucose agar (VRBGA) by counting only the red to dark purple colonies surrounded by a reddish zone (Enterobacteriaceae), after incubation at 37°C for 24 h. The results were expressed as mean log cfu g^{-1} ± standard deviation (sd) of 4 replicates (two replicates per batch).

2.4 16S rRNA meta-barcoding analysis

DNA extraction, quality evaluation and sequencing

Twenty five (25) grams of flesh per batch (N=20 individuals) were used to collect bacterial pellets for 16S rRNA amplicon sequencing, as it was described in Parlapani et al. (2018b). For each sample, 200 μl of each diluted pellet were used for bacterial DNA extraction with ZR Soil Microbe DNA MicroPrep (ZYMO RESEARCH; Irvine, CA, USA). DNA concentration was measured on a Qubit 2.0

Fluorimeter using the Qubit® dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA). The total DNA (DNA from two batches) was pooled before the analysis.

Metagenomic analysis was performed by amplifying the V3–V4 region of the 16S rRNA gene using the Illumina's 16S Metagenomic Sequencing Library Preparation (15044223 B) protocol. For the amplification of the V3-V4 region, gene-specific primers were selected based on the Klindworth et al. (2013), by adding Illumina overhang adapter nucleotide sequences at the 5' end. PCR reactions were performed in a 36-well rotor carousel on a Rotor-Gene Q Thermocycler (Qiagen, Hilden, Germany) according to Parlapani et al. (2018b).

PCR products were purified to remove unincorporated primers and primer-dimer species using NucleoMag® NGS Bead Suspension (Macherey-Nagel, Düren, Germany). A second PCR was performed to attach dual indices and Illumina sequencing adapters in all PCR fragments following the instruction of the Illumina's 16S Metagenomic Sequencing Library Preparation. All libraries were quantified with fluorometric quantification using Qubit® dsDNA BR assay kit and their molarity was calculated in relation to the size of DNA amplicons after indexing. Quantitative PCR (qPCR) was conducted on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) with the KAPA Library Quantification kit for Illumina sequencing platforms (KAPA BIOSYSTEMS, Woburn, MA, U.S.A.).

Sequencing was performed on a MiSeq platform according to the manufacturer's instructions, using the MiSeq® reagent kit v3 (2 x 300 cycles) (Illumina, San Diego, California).

Bioinformatics and Data Analysis

Trim Galore wrapper (Krueger, 2015) was used for quality trimming of raw reads (.fastq files). Sequences were clustered into operational taxonomic units (OTUs) using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010), with 99% sequence similarity to SILVA 128 release for QIIME database. OTU picking was executed using the default clustering algorithm UCLUST (Edgar, 2010). Further processing of OTU tables was conducted in R version 3.4.2 (R Development Core Team, 2010). OTU counts and taxonomic assignments were merged to a phyloseq object with phyloseq R package (McMurdie & Holmes, 2013). Sequences have been submitted through the NCBI sequencing submission portal and deposited under the SRA: SUB6377492, with accession number SAMN12878864.

2.5 VOCs determination by Headspace SPME-GC/MS analysis

Ten (10) g of flesh from approximately ten shrimps were homogenized. Five grams of the homogenized sample in duplicate per batch were transferred into a 20 mL glass vial (two replicates per batch, 2x2=4 replicates in total). The SPME-GC/MS analysis was performed according to Parlapani et al. (2015a). Compounds were identified by comparing: (i) the linear retention indices (LRI) based on an homologous series of even numbered n-alkanes (C8–C24, Niles, Illinois, USA) with those of standard compounds and literature data, and (ii) the MS data with those of reference compounds and that of MS data obtained from NIST library (NIST/EPA/NIH Mass Spectral Library with Search Program, data version NIST 05, software version 2.0d). The Amdis software (version 2.62, http://chemdata.nist.gov/mass-spc/amdis/) was used for the deconvolution of mass spectra and the identification of target components. The amount of VOCs was

expressed in arbitrary units of the peak area of the deconvoluted component multiplied by 10^{-6}.

2.6 Statistical analysis

For the 16S data plots were visualized by combining functions provided by the ggplot2 R package (Wickham, 2009).

Alpha diversity indices were calculated using the Chao1 and ACE indices (Chao & Chiu, 2016) were assessed to measure species richness and the Shannon, Simpson, Inverse Simpson and Fisher indices (Lande, 1996) were used to assess the proportional abundances and frequencies of the identified species by the *vegan* package of R.

Abundance estimations for each species were normalized to 100% within each sample, thus, percentages do not reflect the true biomass fraction of each sample. Venn diagrams were constructed using the InteractiVenn online tool (Heberle, Meirelles, da Silva, Telles & Minghim, 2015) to depict pairwise comparisons of shared, common and/or unique OTUs. Spearman's non-parametric correlations were used through the R package *psych* to study the relationships between microbial taxa abundance and volatilome profile and visualized in R using the *corrplot* package of R.

Differences of means in microbial populations were subjected to Analysis of Variance (ANOVA) followed by Tukey's significant difference test, using STATISTICA 6.0. A probability level of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 Rejection time of chill-stored shrimps

Sensory attributes of shrimps were evaluated as 'excellent' at the beginning of the experiments (T0, Table 1). Their shell presented bright colors. Flesh odor was fresh and marine. At the time of minimum acceptability (grade 3), the shrimp odor was weak "fishy odour", even slight ammonia was produced, and the color was natural light pink with grey-greenish, and the shell lacked luster sheen. After this point, the shrimps exhibited unpleasant odor, thus they were graded as unfit and rejected (Table 1). Therefore, the shelf-life determined by sensory assessment was estimated to be 5 days at 0°C (ice) and 2 days at 4°C.

3.2 Microbial population changes

At the beginning of the shrimp's shelf-life (T0), the APC was found to be 3.12±0.58 log cfu/g, while the populations of *Pseudomonas*, H_2S producing bacteria and LAB were found to be 2.69±0.61, 2.04±0.08, 3.00±0.70 log cfu/g, respectively (Table 2). During storage, the bacteria were grown faster at 4°C reaching 4 to 5 logs for all the population monitored after one day of storage ($p \leq 0.05$, T1, Table 2) and reached up to 6.50 logs after two days of storage (T2). In respect of ice-stored shrimps, APC was found to be 6.59±0.36 log cfu/g after five days of storage (T5), while the populations of *Pseudomonas*, H_2S producing bacteria and LAB reached 6.11±0.53, 4.96±0.63 and 5.23±0.47 log cfu/g respectively (Table 2). Enterobacteriaceae remained below the detection limit of 1 log cfu/g throughout the experiment.

3.3 NGS analysis

3.3.1 Illumina MiSeq data analysis

After sequencing 500,206 reads were obtained and after the quality filtering 465,369 sequences were assigned to 4,026 unique OTUs Table S1. In particular, 90.6%, 90.4%, 89.4%, 88.5% and 85.1% of reads were assigned to the Phylum, Class, Order, Family and Genus level, respectively. The most diverse community was observed for T2-0°C storage conditions in which the highest number of unique OTUs was observed among the studied samples (Table S1). That was also verified by the Chao1 index (Table S2).

3.3.2. Microbiota composition

A simple microbiota was observed in all the samples analysed with the predominance of *Photobacterium* (18.0%) as the main microbes in fresh shrimps (T0), while *Psychrobacter* and *Carnobacterium* dominated together in shrimps during storage at both temperatures (Table 3). *Carnobacterium* was recorded at abundances of 62.4 % at 0° and 20.5% at 4°C at the middle stages of storage, while the *Psychrobacter* at abundances of 34.0 at 0°C and 52.5% at 4°C (Table 3). *Psychrobacter* became the most dominant microorganism at the end of shelf-life of shrimps (57.7% and 48.0% in samples at 0°C and 4°C, respectively), while *Carnobacterium* was also found at 29.8% and 41.2% at 0°C and 4°C, respectively (Table 3). Other genera like *Candidatus Hepatoplasma*, *Pseudomonas*, *Acinetobacter*, *Delftia*, *Brevundimonas*, *Stenotrophomonas*, *Bacillus*, *Vibrio*, *Vagococcus* were also found but at lower abundances (up to 11.3%) in fresh or chill-stored samples (Table 3), while other 167 genera including *Staphylococcus*, *Legionella*, *Bacillus*, *Escherichia-Shigella*, *Enterococcus*, *Enterobacter*, *Klebsiella* etc. were found at abundances of less than 1% in all cases (Table S3).

3.4 VOCs produced in chill-stored shrimps

A total of 41 VOCs were identified (11 alcohols, 12 aldehydes, 11 ketones, 3 esters and other 4 compounds: dimethyl sulfide, methylene chloride, carbon disulfide and indole). By comparing the presence of the VOCs between T0 and samples stored in ice or in refrigerate condition it was possible to observe that samples stored at 4°C were characterized by the presence of ethanol, 3-methyl-1-butanol, 2-ethyl-1-hexanol, *trans*-2-nonenal, *trans*-2,*cis*-6-nonadienal, 3-hydroxy-2-butanone, 3-octen-2-one and indole, while acetone and dimethyl sulfide were mainly associated with ice storage (Fig 1a). The compounds 1-heptanol, 1-nonanol, octanal, 3-pentanone and methylene chloride were associated with the fresh shrimps (T0, Fig 2a). Some compounds were also increased in shrimps stored at 0°C (i.e. 1-octen-3-ol and *trans*-2-octenal) or at 4°C (i.e. 3-methyl-1-butanol, *trans*-2,*cis*-6-nonadienal and indole), while the compound 2-methylbutanal and 3-methylbutanal were increased at both 0 and 4°C (Fig 1b).

3.5 Correlation between Microbiome and Volatilome in rose shrimps

Spearman's correlation between the microbial OTUs and VOCs clearly indicated the correlation of the most abundant bacterial species in rose shrimps with the concentration of metabolites produced during their chill storage ($P < 0.05$, Fig 2). *Psychrobacter* and *Carnobacterium* which found to be the most abundant genera during storage at both temperatures, they were positively correlated with some VOCs. In particular, *Psychrobacter* was positively correlated with 2-ethyl-1-hexanol, 2-nonanone and 2-octanone, and *Carnobacterium* with 3-methyl-1-butanol and 2-pentanone. Positive correlations were also found between *Candidatus Hepatoplasma* and 1-heptanol, 1-nonanol, octanal, 3-pentanone, *Photobacterium* and 2-butanone,

Vagococcus and 3-methyl-1-butanol, 2-methylbutanal, 3-methylbutanal, 2-octanone, 2-nonanone, *Vibrio* and 2-butanone, ethyl acetate (Fig 2). On the other hand, *Psychrobacter* was negatively correlated with dimethyl sulphide, while *Delftia* and *Lactobacillus* with 3-methyl-1-butanol (Fig 2).

4. Discussion

The 16S sequencing coupled with VOCs profile analysis were used to investigate microbial spoilage of the deepwater rose shrimp caught from Greek seawaters. The correlation of the microbiome with volatilome and their link with sensory score allow us to understand how the quality of shrimp is being deteriorated during storage and how it is eventually rejected.

Fresh shrimp remains one of the most delicious and delicatessen food in restaurants, catering and all-inclusive hotels along Greek coasts, inland and islands. However, the shelf-life of the deepwater rose shrimp is short as it was determined in this study (5 days in ice and 2 days at 4°C). *Pseudomonas*, H_2S producing bacteria and LAB are usually responsible for the quality loss of chill-stored shrimp such as the tropical brackish water shrimp *Penaeus notialis* (Dabadé et al., 2015). In the present study, these organisms reached the levels of 5-6 logs at the end of fish shelf-life, with *Pseudomonas* to dominate the shrimp using the classical microbiological approach. However, *Pseudomonas* was found at very low abundances using the 16S NGS analysis (culture independed approach). This indicates that organisms other than *Pseudomonas* might grew on CFC culture medium. The microbiota grown on the selective culture media has to be investigated using molecular methodologies in future studies.

Microorganisms such as *Acinetobacter*, *Bacillus* and *Delftia*, which were found during the first days of storage, are usually associated with the handling conditions and contamination occurs from the human skin, equipment, soil, washing water or ice, etc. (de Amorim & Nascimento, 2017; Heinsz et al., 1988; Parlapani et al., 2018). Other bacteria coming from human and environmental sources like *Enterobacter, Escherichia*, *Shigella, Klebsiella, Legionella, Enterococcus* and *Staphylococcus* are also involved in the inadequate attention to hygiene during fishing, handling and icing affecting fish quality and safety. The fact that these genera were found at abundances less than 1% herein does not minimize the possibility of pathogens presence. All of them contain human pathogens, foodborne or not, might be found in raw shrimps. In that case, a likely consumption of undercooked shrimps could cause foodborne infections causing socioeconomic hardship in the Greek market, like legal penalties, shutdown of business, and tarnished reputation. On the other hand, the detection of them using 16S NGS does not indicate potential risk. NGS is not able to differentiate living and dead cells, thus overestimating the living taxa in microbial communities (Emerson et al., 2017). To minimize contamination in order to maximize the quality and safety of fishery products, the findings derived from this and previous studies (Parlapani et al. 2018a; 2018b; 2019) could be used to develop approaches for rapid analysis like the High Resolution Melting analysis (HRM) or the multiplex PCR using specific genes as targets for the identification of pathogenic species/strains contained into the genera identified using 16S meta-barcoding. In addition, we could strengthen extension activities (education, communication and outreach) for individuals, communities and industries involved in the primary production in Greece.

The 16S data also revealed herein the presence of *Candidatus Hepatoplasma*, *Photobacterium* and *Psychrobacter* at the beginning of deepwater rose shrimp shelf-life. From those, *Candidatus Hepatoplasma* has been described as symbionts of the terrestrial isopods *Porcellio scaber* and *Oniscus asellus* (Wang, Stingl, Anton-Erxleben, Zimmer & Brune, 2004; Wang, Brune & Zimmer, 2007), while the other two bacteria are usually found in fresh fish (Gram & Huss 1996). *Photobacterium* has been also reported as spoiler of fish under particular storage conditions. *Photobacterium* (*P. phosphoreum* or *Photobacterium* sp.) usually predominate in fish such as the cod, the halibut and the cold-smoked salmon under elevated CO_2 concentrations (Modified Atmosphere Packaging – MAP) or vacuum (Gram & Dalgaard, 2002; Hovda, Sivertsvik, Lunestad, Lorentzen & Rosnes, 2007; Kuuliala et al. 2018; Olofsson, Ahrne & Molin, 2007; Stohr, Joffraud, Cardinal & Leroi, 2001). The facultative fermentative metabolism of *Photobacterium* and the competitive interactive environment defined by the presence of other bacteria in fish flesh stored under aerobic conditions (Gram & Dalgaard, 2002) could may explain the absence of its growth increase during the storage of the deepwater rose shrimp. *Psychrobacter* and *Carnobacterium* were observed to co-dominate during storage in this study, at the first two days at 4°C, and the last two days at 0°C. This means that *Psychrobacter* and *Carnobacterium* bacteria dominate the shrimps stored at the higher temperature (4°C), in a shorted time than the ice-stored shrimps (0°C). *Psychrobacter* usually dominate the chill-stored crustaceans like the brown shrimp (Broekaert, Heyndrickx, Herman, Devlieghere & Vlaemynck, 2013a; Broekaert, Noseda, Heyndrickx, Vlaemynck & Devlieghere, 2013b), the Pacific white shrimp (Yang et al., 2017), the Norway lobster (Bekaert, Devriese, Maes & Robbens, 2015), but also the fish like the gilt-head sea bream (Parlapani et al., 2018a), the thawed hake, the thawed plaice fillets (Zotta et

al., 2019) or even the cephalopods like the thawed cuttlefish (Parlapani et al., 2018b). This bacterium might causes the spoilage of the brown shrimp through the break down of lipids and the hydrolysis of proteins and amino acids (Broekaert et al., 2013b). On the other hand, *Carnobacterium* was found to dominate the peeled brown shrimp stored under MAP at 4°C (Calliauw et al., 2016a), or it was detected in shrimps stored in MAP (Dalgaard et al., 2003) or in fish species stored in air (Parlapani et al., 2018a; Zotta et al., 2019).

The compound 3-methyl-1-butanol has been associated with the metabolic activity of *Carnobacterium* in inoculated cooked and peeled tropical shrimp *Penaeus vannamei* stored under MAP at 8°C (Jaffres et al., 2011). In our study, this compound was positively correlated to the presence of the *Carnobacterium* and it was increased in shrimps stored at 4°C. *C. divergens* and *C. maltaromaticum* are known to cause sensory spoilage of cooked and peeled shrimps stored under MAP at 5°C, producing ammonia, tyramine, alcohols (including 3-methyl-1-butanol), aldehydes and ketones (including 2-pentanone, Laursen, Leisner, & Dalgaard, 2006). Other compounds such as the ethanol, the dimethyl sulfide and the ethyl acetate were being increased in cooked peeled gray/brown shrimp *Crangon crangon* stored under MAP (Noseda et al., 2012). The ethyl acetate, which showed a positive correlation with *Vibrio* herein, has been also reported to the brown shrimp (Kuuliala et al., 2018). *Vagococcus* (particularly *V. salmoninarum*) was found to produce 2-nonanone in inoculated brown shrimp stored under MAP at 4°C (Calliauw, Horemans, Broekaert, Michiels & Heyndrickx, 2016b). From the aforementioned compounds, only the 3-methyl-1-butanol increased in this study. Additionally, the 1-octen-3-ol and the *trans*-2-octenal were increased in the deepwater rose shrimps stored at 0°C, the *trans*-2,*cis*-6-nonadienal and indole in those stored at 4°C, while the 2-methylbutanal and the 3-

methylbutanal they were increased in the shrimps stored at both temperatures. The 1-octen-3-ol, *trans*-2-octenal and *trans*-2,*cis*-6-nonadienal are associated with chemical oxidations in fish (Duflos, Coin, Cornu, Antinelli & Malle, 2006; Iglesias & Medina, 2008), while the 2-methylbutanal and 3-methylbutanal are products of microbial activity (Parlapani, Mallouchos, Haroutounian & Boziaris, 2017). The compounds 3-methyl-1-butanol, 1-octen-3-ol, *trans*-2-octenal, 2-methylbutanal, 3-methylbutanal and indole they also exhibited an increasing trend in chill-stored blue crabs and proposed as potential spoilage markers of that product (Parlapani et al., 2019). The indole has been associated with the spoilage of *P. longirostris* (Mendes et al., 2005; Mendes, Huidobro & Caballero, 2002) and it has been found as a product of various bacteria like *Vibrio* spp. and *Morganella morganii* isolated from the wild shrimps *Litopenaeus setiferus* and *Litopenaeus brasiliensis* (Benner, Staruszkiewicz & Otwell, 2004). Herein, no correlation between indole (which found at very low relative concentrations at 4°C) and bacteria was observed. This may occurs since indole is not produced in significant quantities by bacteria in shrimps stored at low temperatures (Mendes et al., 2002). Other compounds such as hexanol, heptanol, hexanal, heptanal, octanal and nonanal found in rose shrimp are known as aroma compounds and are mainly coming from enzymatic reactions, oxidation or autoxidation of lipids (Alasalvar, Taylor & Shahidi, 2005; Duflos et al., 2006).

Overall, the inadequate hygiene during fishing/handling can cause microbial contamination from human, foodstuffs and environmental sources. In this way, the initial total microbial population increases, the quality of fish product is lost rapidly and the product might not be safe to consume. For these reasons, fishermen have to reinforce the Good Hygiene Practices regarding the potential sources of microbial contamination during handling which usually reduce quality and shorten shelf-life of

fish. Moreover, the shrimps have to be stored and distributed in ice (rapid onboard icing and keeping fish iced until marketing or consumption). To reinforce the competitiveness of fishery products at local, national or international commercial level, the research outputs should be communicated through websites, workshops, public dialogues, newsletters, and one-on-one consultations in order to create awareness among academia, fishery sectors and society.

Conclusions

The 16S meta-barcoding revealed microorganisms associated with safety or spoilage of the deepwater rose shrimp from Greek seawaters. The simulated storage conditions showed how the higher temperature causes the fast domination of the shrimps by the spoilers *Psychrobacter* and *Carnobacterium* resulting to their rapid quality deterioration (shelf-life: 2 days at 4°C vs 5 days in ice). In addition, compounds associated with microbial activity e.g. 3-methyl-1-butanol, 2-ethyl-1-hexanol and 2-nonanone presented a positive correlation with the dominant organisms e.g. *Psychrobacter* or *Carnobacterium* in rose shrimp. From them, 3-methyl-1-butanol increased during storage. Other compounds associated with chemical reactions e.g. 1-octen-3-ol, *trans*-2-octenal and *trans*-2,*cis*-6-nonadienal were also increased. This indicates that not only microbial action, but also chemical mechanisms might be responsible for the quality loss of chill-stored rose shrimp. Based on our findings, we could suggest preventive measures to improve the quality and safety of rose shrimp, particularly fishermen have to reinforce the Good Hygiene Practices during fishing and handling, to accomplish rapidly onboard icing and keep shrimps iced avoiding even small changes in storage temperature that affect the microbial population level, synthesis of microbiota, VOCs profile and eventually the shelf-life of the product. The

results of this study will be used to prepare a guide for the proper handling and storage conditions of the fishing products produced by the small-scale fishery sector in Greece in order to provide a product of the highest quality and safety in the market.

Acknowledgments

This research has been co-financed by the European Union (European Social Fund — ESF) and the Greek government through the Operational Program "IKY Scholarship Programs for Strengthening Post Doctoral Research" in the frame of the Human Resources Development Program, Education and Lifelong Learning.

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Table 1. Sensory score of deepwater rose shrimp stored at 0°C (ice) and 4°C.

	0°C (ice)				4°C	
Sensory score	**T0**	**T2**	**T4**	**T5**	**T1**	**T2**
Shell appearance	Excellent	Good	Acceptable	Unacceptable	Acceptable	Unacceptable
Flesh odor	Excellent	Excellent	Good	Unacceptable	Good	Unacceptable

Table 2. Population changes (mean log cfu g^{-1} ± sd) of four replicates during storage (in days, T) of shrimps stored at 0°C (ice) and 4°C.

	0°C (ice)				4°C	
	T0	**T2**	**T4**	**T5**	**T1**	**T2**
APC	3.12 ± 0.58^{a}	4.32 ± 0.38^{b}	5.92 ± 0.38^{c}	6.59 ± 0.36^{d}	4.85 ± 0.62^{b}	6.44 ± 0.46^{c}
Pseudomonas	2.69 ± 0.61^{a}	3.44 ± 0.43^{b}	5.39 ± 0.46^{c}	6.11 ± 0.53^{d}	4.46 ± 0.51^{e}	5.97 ± 0.47^{c}

H_2S bacteria	2.04±0.08[a]	2.82±0.57[b]	4.14±0.73[c]	4.96±0.63[d]	4.15±0.49[c]	5.14±0.10[d]
LAB	3.00±0.70[a]	3.69±0.85[a]	4.43±0.33[b]	5.23±0.47[c]	4.29±0.61[b]	5.08±0.48[c]

Mean values with different letters are significantly different ($p<0.05$). Mean population values along different time (T0-T5) or temperature values (0 and 4°C), are compared using *ANOVA*.

Table 3. The Relative abundance (%) of the dominant genera assigned to 16S rRNA sequences, in the deepwater rose shrimp stored in 0 and 4°C.

			0°C (ice)		4°C	
OTUs (%)	**T0**	**T2**	**T4**	**T5**	**T1**	**T2**
Psychrobacter	8.10	20.9	34.0	57.7	52.5	48.0
Carnobacterium	1.60	8.70	62.4	29.8	20.5	41.2
Pseudomonas	0.30	7.30	0.60	0.70	11.3	0.60
Photobacterium	18.0	0.10	0.00	0.00	0.00	0.20
Acinetobacter	5.20	0.90	0.20	5.30	0.10	0.10
Delftia	4.20	6.70	0.10	0.10	0.10	0.00
Candidatus Hepatoplasma	10.0	0.00	0.00	0.00	0.00	0.00
Vagococcus	0.00	0.00	0.60	2.00	1.90	5.30
Stenotrophomonas	1.10	7.00	0.00	0.20	0.20	0.30
Brevundimonas	6.50	1.50	0.10	0.30	0.10	0.10
Bacillus	0.30	6.90	0.00	0.30	0.00	0.10
Vibrio	5.40	0.00	0.00	0.00	0.00	0.20
Exiguobacterium	0.10	0.00	0.00	0.00	4.40	0.00
Lactobacillus	4.30	0.10	0.00	0.00	0.10	0.00
Paucimonas	1.50	2.20	0.00	0.10	0.00	0.00
Planococcus	0.10	3.20	0.00	0.30	0.20	0.10

Rhizobium	1.00	2.30	0.00	0.10	0.10	0.00
Aerococcus	0.10	0.20	0.00	0.10	2.60	0.30

Legends to Figures

Figure 1. Panel A. Abundance of VOCs (relative concentrations, area x 10^{-6} under the chromatographic peak) during storage of fresh shrimp (T0, red bars) at Time stored in ice (**green bars)** or 4°C (**blue bars). Panel B. Shift in volatilome profile** during storage at different storage conditions (T2, T4 and T5 for ice-stored shrimps and T1_4°C and T2_4°C for shrimps stored at 4°C). Boxes represent the interquartile ranges (IQRs) between the first and third quartiles, and the lines inside represent the medians (2nd quartiles). Whiskers denote the lowest and the highest values within IQRs from the first and third quartiles, respectively. Circles represent outliers beyond the whiskers.

Figure 2. Spearman's correlation between the bacterial OTUs and VOCs in deepwater rose shrimp. The intensity of the colors indicates the degree of correlation between the bacterial OTUs. The blue color shows a positive correlation, while the red colour shows a negative correlation between OTUs and VOCs.

FIG 1-2 (attached file)

780

Figure 1a

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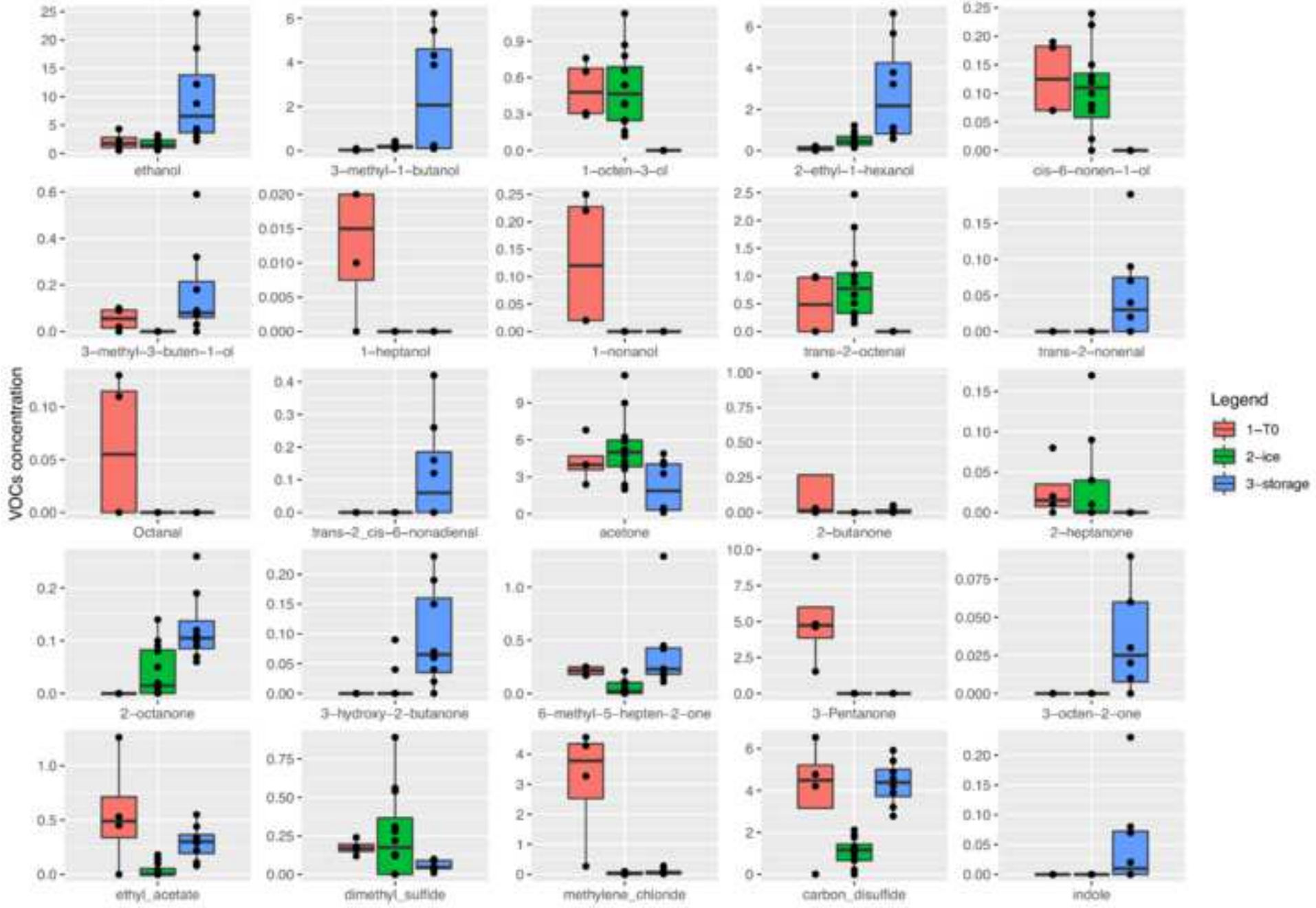

Figure 1b

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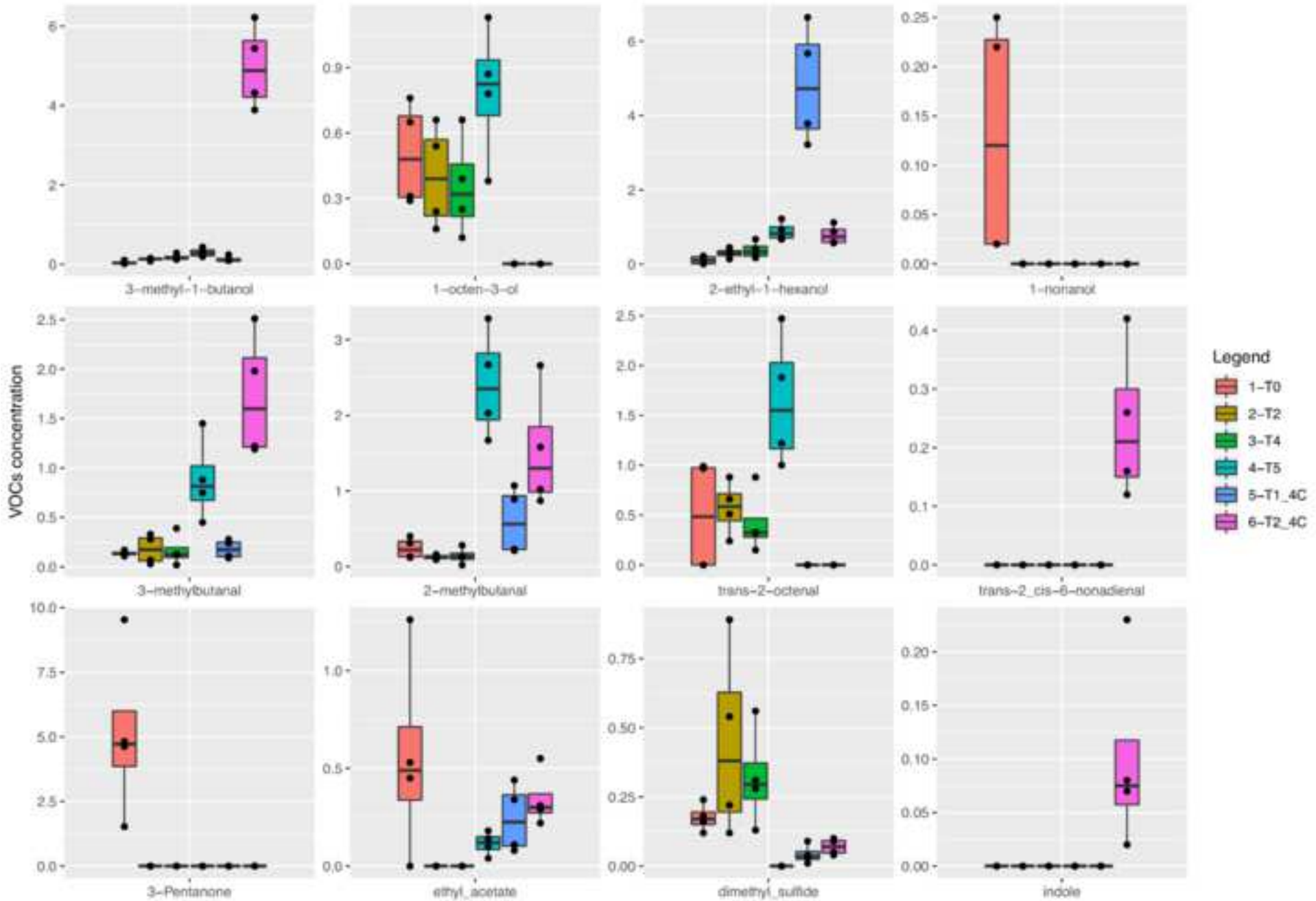

Figure 2

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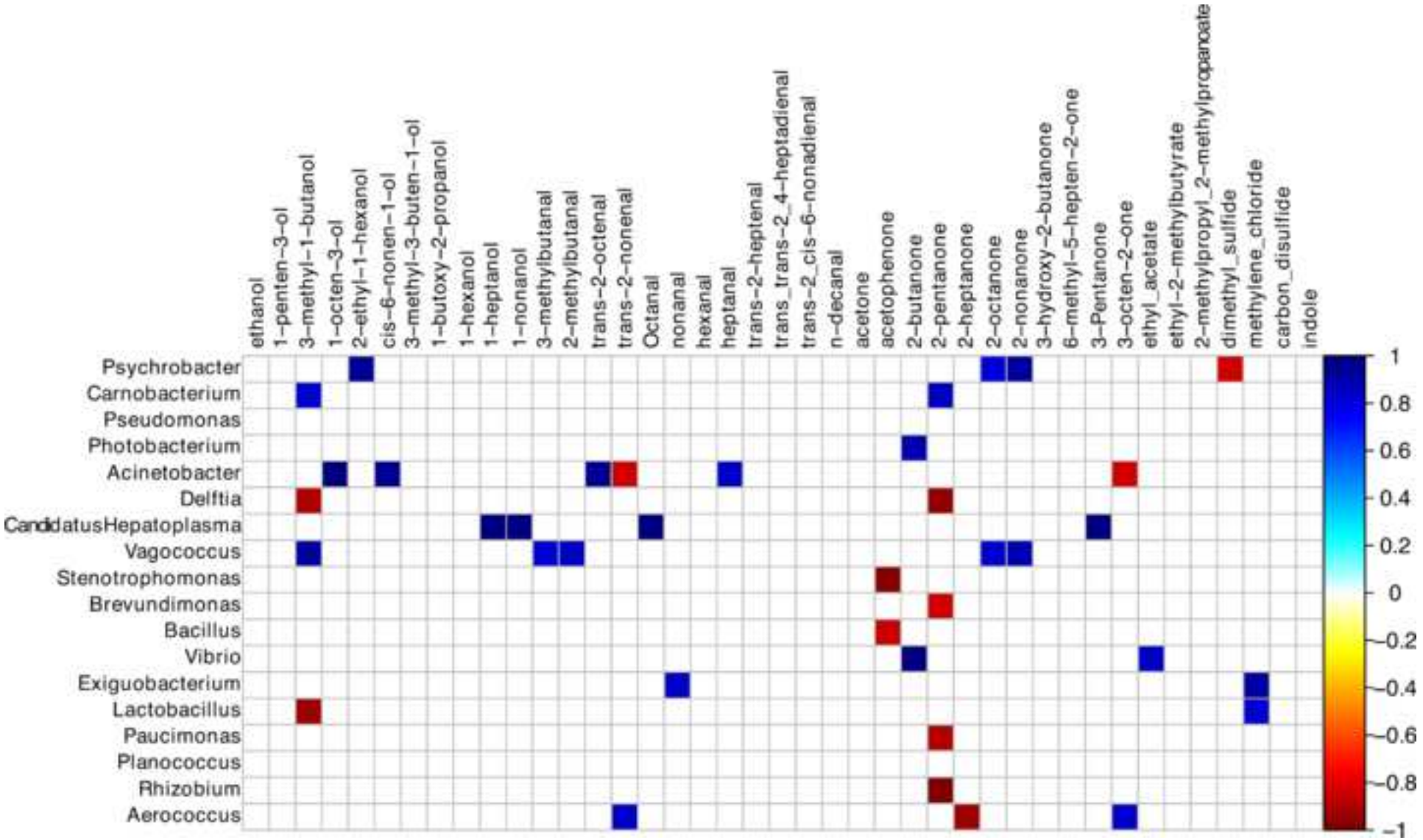

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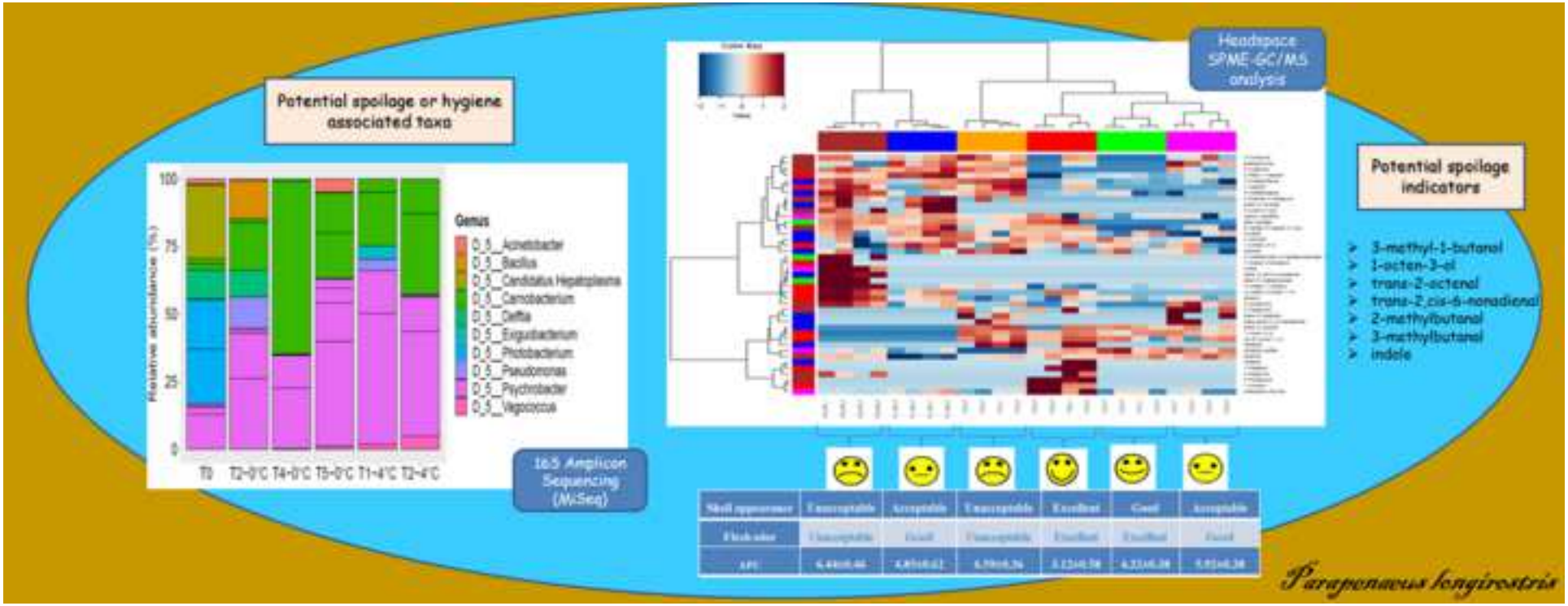

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Ilario Ferrocino: Methodology, Formal analysis, Investigation, Writing-Review and Editing

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***Conflict of Interest form**

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Volos, 05-10-2019

Dear Editor,

authors declare no competing interests.

Sincerely Yours,

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